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The ESCRT-machinery: closing holes and expanding roles

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Abstract

The ESCRT machinery is an ancient, evolutionarily conserved membrane remodelling complex that is deployed by cells to perform a diverse collection of physiological and pathophysiological processes. ESCRT proteins are needed for multivesicular body biogenesis, release of enveloped retroviruses, reformation of the nuclear envelope and cytokinetic abscission during mitotic exit. These events all share the requirement for a topologically equivalent membrane remodelling for their completion that is thought to be performed by ESCRT-III. More recently, ESCRTs have been shown to play an essential roles in repairing damaged cellular membranes, so preserving cellular viability and organellar function. Here, we will examine new advances in our understanding of the cell biology of this fascinating cellular machinery.

Introduction

The Endosomal Sorting Complex Required for Transport (ESCRT) machinery [Table 1] is an evolutionarily conserved multi-subunit membrane remodelling complex. Whilst these proteins were discovered originally for their role in regulating degradative endosomal sorting, the surprise finding of recent years is the cellular employment of this machinery in a variety of essential roles that contribute to normal physiology and pathophysiology. ESCRTs, it seems, really are everywhere [1]. For a detailed analysis of ESCRT biology and function, we refer readers to recent excellent in depth reviews [2,3] and we will explore here some more recent advances in this exciting and fast-moving field.

How do ESCRT proteins remodel membranes?

The ESCRT complex is responsible for many membrane remodelling processes essential for cell physiology including intraluminal vesicle (ILV) formation, viral budding, cytokinetic abscission, exosome release, and repair of membrane fenestrations. These processes all share the requirement for the scission of a cytoplasm filled membranous stalk for their completion (Figure 1A and Figure 1B) [1-3]. Assembly of the ESCRT machinery proceeds as a recruitment cascade originating with site-specific adaptors such as endosomal proteins and ubiquitinated cargoes for MVB biogenesis, midbody components for cytokinesis or viral Gag proteins for retroviral egress. These components trigger recruitment of early-acting ESCRT factors, such as ALIX for cytokinesis, or ESCRT-I/ESCRT-II for MVB biogenesis, which concentrate cargoes, initiate membrane bending and nucleate the assembly of ESCRT-III components. ESCRT-III proteins form a membrane-interacting oligomeric filament that is thought to operate the membrane remodelling event, eventually resulting in scission [2-4]. The biological reasons behind the evolution of 8 different ESCRT-III subunits (with additional homologues present in humans bringing the total number to 12, see Table 1 and Figure 1C) remain unknown. However, some ESCRT-III subunits are recruited only to a site-specific filament, e.g. CHMP7 is thought to nucleate the filament for nuclear envelope resealing [5]. Studies in *Saccharomyces cerevisiae* and studies of HIV-1 release showed that the indispensable components of the filaments are the ESCRT-III proteins Snf7/CHMP4, Vps24/CHMP3, and Vps2/CHMP2 and the hexameric AAA ATPase, Vps4, that is proposed to drive membrane remodelling through a variety of mechanisms [6-8].

ESCRT-III subunits are thought to adopt two distinct conformations, referred to as 'open' or 'closed'. These were believed to represent membrane-adsorbed or soluble conformations, with transition between the two states regulated by displacement of a C-terminal regulatory region (Figure 1C) [9]. The structure of the ESCRT-III filament has recently been visualised in three different approaches, but many details remain unclear. McCullough et al. used a cryo-EM approach to generate a 4 Å resolution reconstruction of a heteropolymeric filament of open CHMP1B with the closed-conformation N-terminus of IST1 [10]. This architecture clarified how ESCRT-III spiralling structure could stabilise membranes with different degrees of curvature. Whilst we commonly associate ESCRT-III-activity with 'reverse topology' membrane scission, this structure revealed that the CHMP1B/IST1 heteropolymer was able to coat 'normal topology' membrane tubules [10], suggesting that the ESCRT-III filament may be more plastic than originally thought. Two other structures of the filament elucidated the similar intermolecular packing mechanisms of the activated filament-forming Snf7/CHMP4 protein [11] and its fly homologue Shrub [12] indicating that subunits polymerized into a staggered, nested array. Both structures agreed on the intermolecular interactions required for the polymerisation, however these truncated CHMP4 versions lacked the C-terminal regulatory region and Vps4-interacting motifs, and resulted in linear crystal packing (while *in vivo* they bind to negatively curved membranes). How other ESCRT-III subunits interact to perform the membrane remodelling remains to be established. The last approach used a combination of electron microscopy and atomic force microscopy (AFM) to study the mechanical properties of Snf7/CHMP4 filament on supported lipid bilayer [13,14]. Curved Snf7/CHMP4 filaments grew radially on supported membranes so to act as a 'spiral spring' with the stored elastic energy increasing in the growing filament as growth forced the adoption of non-preferred curvatures, with this energy proposed to remodel membranes [14]. In this scenario, the activity of Vps4 contributed to constant turnover of ESCRT-III subunits within the growing filaments (both of Snf7 and of Vps2/Vps24 to enable effective filament growth [13]. In recent work by Hurley and Bustamante laboratories [4], a minimal ESCRT-III machinery was encapsulated inside giant unilamellar vesicles and optical tweezers were applied to pull lipid nanotubes to create a reverse-curvature topology in which to visualise ESCRT-III activity. This technique allowed the measurement of the forces generated by Snf7/CHMP4, Vps2/CHMP2, and Vps24/CHMP3 in the presence of Vps4 and ATP confirming that (i) the full set of proteins and ATP were necessary and sufficient for force

generation within the tube; and (ii) active Vps4, visualised in fluorescent ESCRT-III/Vps4 puncta along the tube, and consequent ATP hydrolysis were essential to induce nanotube scission. These data provide the first topologically correct *in vitro* reconstitution of ESCRT-III activity and we hope future combinations of biophysical approaches with minimal system reconstitutions and ultrastructural microscopy will enable full understanding of the composition and dynamics of the ESCRT-III filament, as well as the structural and mechanical bases of force generation.

Kinetics and dynamics of ESCRT assembly

New insights into the behaviour of ESCRT complexes on endosomes

ESCRT activity is the only mechanism by which transmembrane cargo that has escaped the secretory pathway can be degraded. Understanding the biology of ESCRT function on endosomes has proved a rich arena. Technological advances have provided greater insight into the behaviour of these proteins on endosomes and a number of high spatial and temporal resolution studies have helped us understand how this machinery acts. Whilst ILV-containing multivesicular endosomes (MVEs) are commonly thought to be 'late' endosomes, Raiborg and colleagues showed recently that ESCRT components were recruited within 2-5 minutes of stimulation, and showed maximal colocalization within 15 minutes of stimulation, suggesting that degradative fate is encoded early in the endosomal lifecycle [15]. Rather than being constantly present on endosomes, waves of early ESCRT-0 or ESCRT-I recruitment, and transient waves of later-acting ESCRT-III components were observed on the same endosome. These data suggesting that multiple rounds of ILV biogenesis occur at the same site and that there is temporal coordination between cargo-sorting and membrane fission activities. Correlative light and electron microscopy suggested that each ESCRT recruitment wave resulted in the generation of individual ILVs, indicating that repeated recruitment waves would permit the sequential generation of multiple ILVs within a single endosome (Figure 2) [15]. Consistent with this, in worms [16] and plants [17], individual ILVs have been observed to be concatenated, suggesting that they bud continuously from stable endosomal microdomains. Concatenated ILVs were stable and the concatenations themselves appear sufficient to restrict diffusion of membrane bound cargo back to the endosomal limiting membrane without requiring scission [17]. Whether a proteinaceous tether acts to stabilise these concatenations is unclear, but persistent ESCRT-III in the neck would be ideally placed

to perform this function. These data tell us that the terminal membrane fission event thought to release vesicles need not actually occur with each round of budding and may speak to an intrinsic inefficiency in the fission process. This can best be appreciated by examining the ESCRT-dependence of HIV-1 release. Using an elegant cytosolic pH cycling system to temporally pinpoint virion and cytosol separation, Simon and colleagues have shown recently that not all virions are released upon the 1st round of ESCRT-recruitment and that multiple waves can be necessary to decouple virion content from cytosol [18]. Tellingly, in these studies, total internal reflection microscopy imaging revealed that ESCRT-III and VPS4 disappeared from the site of fission prior to virion release [18]. These data indicate that ESCRT-III's role is to stabilise a narrow neck, but need to get out of the way before fission occurs, suggesting that they may be blocking formation of a hemifusion intermediate within the neck. Perhaps, in the context of concatenated vesicles, the next wave of budding occurs before ESCRT-III is properly removed.

To investigate the dynamics and assembly of individual ESCRT-III components upon endosomes, Teis and colleagues employed lattice light sheet (LLS) microscopy of yeast bearing endogenously tagged ESCRT components. Rather than being recruited after ESCRT-III, as predicted from previous studies and the expectation that Vps4 acts as a disassembly factor, Vps4 and ESCRT-III were found to be temporally co-recruited [19]. These data indicate that endosomal ESCRT-III assemblies were remodelled by Vps4 throughout the ILV-generation process and that Vps4 is doing more than just recycling this machinery. Endosomal recruitment events were surprisingly transient, lasting between 3 and 45 seconds, however, the majority of these events were deemed 'unproductive' as they were observed to occur in the presence of ATPase-defective Vps4, and upon endosomes too small to permit ILV generation. A subset of recruitment events accumulated 3 or more Vps4 hexamers and up to 200 Snf7 molecules, and were posited to represent productive ILV biogenesis events [19]. These data have parallels with recent biochemical and cell biological findings showing Vps4 acting to exchange monomers throughout the growth and remodelling of an ESCRT-III filament [13]. Using high-speed AFM, Roux and colleagues observed that co-polymers of Snf7 and Vps2/Vps24 actually assembled *in vitro* as separate intertwined homotypic spirals [13], consistent with the observed co-recruitment of Vps24, Snf7 and Vps4 from LLS data [19]. These data argue against the behaviour of Vps2/Vps24 as a capping subunit and Vps4 as a

terminal disassembly factor and suggest that these proteins may act together throughout the ILV biogenesis cycle. Thus, rather than a stochastic activity on later endocytic structures, cargo presence on early endosomes templates the biogenesis of a 'degradative domain' from where repeated cycles of ILV generation can occur and can, in some cases, can lead to the generation of concatenated ILVs. Cargo-concentrating ESCRT-0 and -I complexes are recruited first, followed by transient co-recruitment of ESCRT-III subunits and Vps4. If sufficient ESCRT-III and Vps4 molecules are recruited, a productive budding event will ensue (Figure 2).

ESCRTs on endosomes – integration with other machineries

ESCRTs aren't the only PtdIns(3)P-coordinated sorting machinery on endosomes. As well as degradative cargo sorting to the ILV, retromer and the sorting nexins act to extract cargo from these organelles for recycling back to the plasma membrane, or retrieval to the Golgi [20]. Whilst ubiquitination is a dominant signal for ESCRT-dependent degradation, it is not obvious how this pathway is coordinated with recycling or retrieval pathways. Although it is a key initiator of degradation, the ESCRT-0 component HRS localises the actin nucleator WASH to endosomes [21]. Endosomal actin can support the generation of sorting-nexin-derived tubulovesicular carriers exiting endosomes for cargo recycling, and an actin-binding motif countered ubiquitination to regulate the balance of recycling versus degradation at this organelle [21]. In worms, removing retromer allows ESCRT coverage to expand on endosomes leading to the inappropriate degradation of cargoes that are usually recycled [22], suggesting that entry into this degradative pathway may not be as regulated as we once thought. Endosomes from cells lacking the ESCRT-III component IST1 display more recycling tubules and it was found that IST1-dependent recruitment of Spastin acted to sever these tubules from endosomes [23]. In recent years, it has been shown that this occurs at contact sites with the ER [24]. These data suggest that the sorting machineries that regulate these trafficking decisions do not act in as isolated manner as we may think, that cytoskeletal elements such as actin may help control the balance between recycling and degradation. Future research efforts ought integrate the biology of sorting complexes acting at these small organelles.

ESCRTs in cell division

Membrane abscission during cytokinesis

ESCRTs are critical mediators of the abscission step of cytokinesis [25-27] and the discovery that this pathway operates in archaea [28] suggest an ancestral role for this machinery in cell division (Figure 3). Mimicking recent discoveries of a contributing role for ESCRT-II in HIV-1 release [29], it appears that cells have two alternate routes for assembling ESCRT-III at the midbody downstream of the midbody component CEP55 – one relies upon ALIX as an adaptor protein between CEP55 and ESCRT-III [26,30] and the other involves an link via ESCRT-I, -II and CHMP6 [31,32]. In recent years, progress has been made in visualising the dynamics and regulation of ESCRT assembly at the midbody. Using cryo-electron microscopy [27], and soft X-ray tomography [33], telling spirals have been observed in the midbodies of cells in cytokinesis, leading to the speculation that contractile ESCRT-III rings narrowed the midbody to a point of fission. Using 3D-STORM and illuminating endogenous ESCRT-III components, the Elia lab have now documented the existence of helical ESCRT-III filaments and characterised their changes during cytokinetic progression; from the initial 2-ring stage where a large 1 µm diameter ring assembles adjacent to the dark zone, to progressively narrowing ring-like structures that appeared in some cases to be connected as a narrowing spiral [34]. As observed previously [35,36], the generation of a secondary pool of ESCRT-III rings was observed at the abscission site. These data suggest that ESCRT-III constriction mediates the membrane remodelling required for cytokinesis by narrowing the midbody through the creation of helical filaments on the inside of the midbody membrane. Whilst ESCRTs may constrict the midbody membrane, it is becoming clear that for this to happen, cortical actin within the midbody must also be remodelled. In this manner, there appears a balance between formin-mediated actin polymerisation and capping protein (CP)-mediated suppression of filamentous actin that is required for proper cytokinesis; in the absence of CP, persistent F-actin led to midbodies that failed to narrow and properly assemble ESCRT-III into ring like structures for division [37]. Midbody actin is also depolymerised through the action of MICAL1, an enzyme that catalyses oxidation of methionine residues in actin [38]. MICAL1 is recruited and activated by midbody Rab35 and MICAL1-depenent actin depolymerisation is necessary for the formation of the secondary pool of ESCRT-III at the site of abscission. However, it is also to be noted that RhoA and Citron Kinase-dependent F-actin has been reported to stabilise these secondary ingressions for ESCRT-III dependent abscission [39], highlighting the need to understand the complex dynamics of actin at these sites. Future

research will hopefully integrate actin, microtubules, ESCRTs and other filament forming machineries such as the septins into a general model for abscission.

Control of the abscission checkpoint

Although cells must complete cytokinesis for effective cell division, there exists an Aurora B-dependent abscission checkpoint [40] that operates through the centralspindlin component MKLP1 [40] and phosphorylation of CHMP4C [41,42] to retard abscission. A variety of stimuli can engage this checkpoint, including defects in chromosome segregation, impaired NPC assembly and even tension at the midbody [43] and it is hypothesised that engagement of this checkpoint gives cells more time to resolve these problems before completing the division process. Alongside Aurora B, other kinases including ULK3 [44], the CLKs [45] and the ESCRT-associated protein ANCHR [46] play roles in checkpoint engagement. Mechanistically, this checkpoint appears to operate by suppressing ESCRT-III polymerisation and retaining VPS4 within the central region of the midbody [41,46]. Cell division checkpoints act typically to protect the genome from chromosome segregation errors and subsequent aneuploidy. In this regard, CHMP4C has been identified in a GWAS as a susceptibility locus for ovarian cancer [47] and a single nucleotide polymorphism (rs35094336) in CHMP4C that is associated with a number of cancers encodes a non-synonymous mutation in CHMP4C (A232T). This mutation impairs interaction with ALIX and cells bearing CHMP4C^{A232T} are checkpoint blind and accumulate DNA damage, chromosome segregation errors and aneuploidy [48]. These data suggest a molecular explanation for the cancer susceptibility of this polymorphism. CHMP4C has also been proposed to localise to unattached kinetochores and to participate in proper chromosome segregation by controlling the mitotic spindle assembly checkpoint (SAC) [49], which could provide an alternate explanation for the involvement of this protein in cancer susceptibility. However, CHMP4C knockout cells displayed an intact SAC [48] and this and previous studies did not report gross chromosome mis-segregation upon CHMP4C depletion [41], suggesting that that involvement of this protein in mitotic checkpoints warrants further study.

ESCRTs at centrosomes

ESCRT-depletion has been reported to alter both centrosome and spindle pole numbers [26], suggesting previously unappreciated roles for these proteins in regulating these membrane-less organelles. VPS4 has been localised to these structures [50,51] and an ATPase defective form of VPS4 reduced γ-tubulin levels, impaired centrosome dynamics and compromised ciliogenesis. Whilst the ATPase activity of VPS4 was needed, ESCRT-III was dispensable for these functions, suggesting that VPS4 may remodel non-ESCRT-III proteins to effect centrosome dynamics and so regulate cell and tissue organisation.

ESCRTs at the nuclear envelope

ESCRTs also operate at the nuclear envelope in a brief 2-4-minute window during mitotic exit. Here, they seal small holes in the reforming nuclear envelope and coordinate this with removal of spindle-microtubules that are enveloped by ER-sheets as they wrap daughter nuclei [5,52]. Just as specific adaptors recruit ESCRT-III to endosomes (HRS/ESCRT-0), the midbody (CEP55) and sites of viral budding (viral L-domains), during division a specific adaptor recruits ESCRT-III to the reforming nuclear envelope. In this case, the adaptor is CHMP7 [5], a hybrid ESCRT-II/ESCRT-III-like protein that harbours an ER-localising and membrane-binding motif in its N-terminal VPS25-like domain [53]. CHMP7 is recruited by LEM2 (Heh1 and Heh2 in yeast) and is essential for localising downstream ESCRT-III components to this organelle [53-55] to effect membrane sealing and nuclear compartmentalisation. Additionally, through IST1, ESCRT-III is able to recruit the microtubule severing enzyme, Spastin, to depolymerise microtubules and coordinate spindle disassembly with membrane sealing [5]. ESCRT-III thus plays an essential role in both generating and maintaining nucleocytoplasmic compartmentalisation and for protecting the genome from cytoplasmic insults. More recently, a role for the CHMP4-binding protein CC2D1B has been observed in the regulation of ESCRT-III activity at this site. CC2D1B is part of the Lgd/CC2D1 family of proteins that binds and controls polymerisation of CHMP4 proteins [56,57]. CC2D1B binds both CHMP7 and, via a C-terminal C2-domain, the membrane lipid PtdIns(4,5)P_2, and assembles at the reforming nuclear envelope in a CHMP7-dependent manner [58]. In the absence of CC2D1B, premature and temporally mal-coordinated ESCRT-III and Spastin recruitment at the reforming nuclear envelope is observed, suggesting that this protein ensures timely polymerisation of ESCRT-III at this organelle, necessary for proper nuclear envelope regeneration.

Open mitoses give organisms opportunity to assess the quality of their nuclear pore complexes, long lived structures that are built for life and that regulate nucleo-cytoplasmic compartmentalisation and exchange. Organisms such as *S. cerevisiae* undergo a closed mitosis and employ ESCRT-III as a surveillance mechanism to sequester defective NPC components preventing them short-circuiting the normal transport activities at the nuclear envelope [59]. The exact mechanism of this surveillance and extraction is the subject of active study, but may involve sealing of a double membrane over the defective NPCs in an ESCRT-III-dependent manner [60]. These data nicely rationalise the disparate ESCRT functions at this organelle, but yeast undergoing a closed mitosis are not the only cells that need to survey their NPCs – quiescent and terminally differentiated cells will no longer have a mitotic opportunity to address problems in compartmentalisation. Using an elegant Cre-mediated switch of tagged protein expression to track old and new proteins in quiescent C2C12 muscle cells, Hetzer and colleagues recently showed that ESCRT-III-dependent whole NPC turnover occurs to replace these complexes [61], suggesting an evolutionary conservation of ESCRT-III-dependent NPC surveillance from yeast to mammals.

In interphase cells, ESCRT-III also repairs nuclear envelope ruptures that occur during constrained migration, helping to maintain cellular viability and protection of the genome [55,62,63]. More recently, it has been shown that viruses that need to cross this membrane employ nucleoplasmic ESCRT proteins to bud across the inner nuclear membrane (INM) and then subsequently fuse with the outer nuclear membrane to effect traversal of this double membrane barrier. ESCRTs had been previously implicated in the lifecycle of herpes viruses such as Epstein Barr Virus (EBV) as the viral protein BFRF1 recruited ALIX and other ESCRT-III proteins to the nuclear envelope [64]. More recently, ALIX and ESCRT-III, but not CHMP7, were shown to be necessary for Herpes Simplex Virus-1 (HSV-1) nuclear export [65]. HSV-1 UL34, the positional orthologue of EBV BFRF1, bound directly to ALIX, recruited downstream ESCRT-III proteins and was necessary for budding of HSV-1 across the INM. Interestingly, removal of ESCRT proteins led to INM expansion in cultured cells, suggesting that ESCRT participates in a continual remodelling of this organelle [65]. ESCRTs thus play important roles in controlling membrane integrity and organellar homeostasis at the nucleus and may participate in the NPC-independent transit of ribonucleoprotein particles across these membranes [65,66].

ESCRTs as controllers of membrane integrity

As well as regulating degradative cargo sorting and membrane remodelling during cell division, an essential role for ESCRT-III in controlling membrane integrity is emerging. For many years, we have known that ESCRT-III can repair damaged regions of plasma membrane and so preserve cellular viability [67,68]. Plasma membrane damage can be induced by interaction with the microenvironment or other external forces. However, it has now become clear that pathophysiological processes can also induce plasma membrane damage that is repaired in an ESCRT-dependent manner [69,70] (Figure 1A). Surprisingly, necroptosis and pyroptosis, forms of programmed cell death, were both antagonised by ESCRT-III activity. During pyroptosis, caspase-dependent cleavage of cellular gasdermin-D creates a pore-forming fragment that can permeabilise the plasma membrane [69]. During necroptosis, mixed lineage kinase like (MLKL) activation can trigger plasma membrane disruption [70], and ESCRT-III-dependent membrane repair acted to both limit pyroptotic cell death and prevent release of further inflammatory mediators. It is hypothesised that pore-containing regions of plasma membrane are shed in ectosomes in an ESCRT-dependent manner. The biology of non-viral release of plasma membrane buds is poorly understood, but we know that ESCRT proteins can assemble here [71,72]. In the case of MLKL activation, ectosomes containing externalised phosphadidylserine were released in an ESCRT-dependent manner [70], suggesting this as a mechanism to antagonise MLKL-dependent necroptosis.

Having initiated a cell death programme, it is not immediately clear why cells would choose to step back from the brink and choose life once more. However, upon removal of MLKL activation stimuli, this is exactly what was observed and perhaps represents and adaptive response to a suicide signal, allowing resuscitation should it be required. Necroptosis, unlike apoptosis, is an immunogenic cell death and, in the case of MLKL-activation, ESCRT-dependent repair gave cells time to alter their transcriptome, synthesise and secrete inflammatory cytokines and chemokines to facilitate cross priming of CD8+ T-cells to alert and activate the immune system [70]. So, whilst ESCRT proteins have a variety of roles in normal physiology, they also play new and important roles in protection from pathophysiological insults that trigger pathways such as necroptosis.

It isn't just the plasma membrane that can get damaged though, cells are constantly sampling their microenvironment, internalising extracellular content and trafficking it to lysosomes for degradation and release of nutrients. However, some internalised materials such as uric acid crystals, asbestos fibres, or lysosomotropic agents, can damage endosomes and compromise endosomal function. Many pathogens access the cytosol by disrupting endosomal membranes, whereas other acidophilic pathogens want to maintain endosomal integrity so that they can replicate effectively in the lysosome. As such, maintaining endosomal and lysosomal integrity is an essential physiological process, and one that is exploited by pathogens. Hanson and Stenmark labs have shown recently that upon addition of lysosomotopic agents that can damage endolysosomal membranes, ESCRT-III components were rapidly recruited to endosomes and acted to repair these holes [73,74]. Unrepaired holes allowed the recruitment of cytosolic galectins, permitting recognition by the autophagy machinery and subsequent organellar degradation. As such, ESCRT-III acts in this context as a molecular puncture repair kit, mending small holes in damaged organelles, preserving their function. ESCRT-III thus has an unexpected role in promoting cell survival and, as well as sorting cargo for lysosomal degradation, provides an activity to ensure that these organelles are competent to degrade cargo. The recruitment signal for this repair activity is currently unclear, but paralleling pathways of ESCRT-III recruitment during cytokinesis, TSG101 and ALIX provide redundant mechanisms to assemble ESCRT-III on damaged endolysosomes [73,74]. However, ESCRT-III recruitment to effect lysosomal repair was wortmannin insensitive [73] and independent of HRS [75], suggesting that an alternate endolysosomal adaptor upstream of ALIX and TSG101 exists to orchestrate this recruitment. ESCRT-III recruitment to these sites of damage has been described to be calcium dependent [73,75] and the calcium-binding protein ALG2 has been previously implicated in recruitment of ESCRT-III to effect repair of regions of damaged plasma membrane [68,76]. However, alternate reports suggest the calcium independence of this endosomal repair pathway [74,77] and whether ALG2 represents this adaptor awaits experimental validation.

A number of pathogens usurp the endosomal system to access the cytoplasm by using the low pH to trigger endolysosomal rupture. *Mycobacterium tuberculosis* employs a Type VII secretion system (T7SS) to rupture endosomal membranes and allow cytosolic replication. ESCRT-III is recruited to endosomes damaged by *M. tuberculosis*, and disrupting the T7SS to

prevent membrane damage abrogated this recruitment [75]. *M. tuberculosis* injects secreted effectors through its T7SS to modify host-cell biology and enable replication. Two of these proteins, a heterodimer of EsxG and EsxH, interact with HRS [78]. Although recruitment of ESCRT-III to damaged endolysosomes was independent of HRS, EsxG/EsxH could impair ESCRT-III assembly at sites of endolysosomal damage in *M. Tuberculosis* infected cells and in situations of sterile damage [75]. A related endomembrane repair function for ESCRTs was revealed upon infection of *Dictyostelium discoideum* with the mycobacteria *Mycobacterium marinum*. Here, TSG101, CHMP4 and VPS4 were recruited to sites of phagosomal endomembrane damage induced by *M. marinum*'s ESX1 secretion system [77].

Interestingly, pathogens entering cells through the endosomal system need to escape before they are degraded by the acidic lysosome [79]. Antagonism of ESCRT function may provide access to the cytoplasm, but will also neutralise endolysosomal acidification to prevent pathogen degradation in this compartment. Other pathogens, such as *Coxiella burnetii* actively seek an acidic endolysosomal environment in which to replicate. Compromising ESCRT-dependent endolysosomal repair impaired *C. burnetii* replication, suggesting that some pathogens exploit this repair pathway to create an environment that actually favours their replication [74].

ESCRTs are typically thought to close holes in double membraned organelles, and the topology of membrane damage in a single membraned organelle, such as the endosomal limiting membrane or the plasma membrane, creates a topological quandary (Figure 3i). It has been hypothesised that budding of damaged regions out of the plasma membrane may provide the topology for ESCRT-III to work on, but it is not obvious how these budded structures would be generated. Perhaps an analogous situation occurs at endosomes where damaged regions are incorporated into budding ILVs to take advantage of this natural process (Figure 3ii)? Reports of pathogen-induced damage causing the budding into vacuoles and extrusion of macroscopic regions of damaged plasma membrane suggest that canonical ESCRT-III-mediated budding may indeed effect removal of these damaged regions [77]. However, it is not immediately obvious that torn or damaged membranes could support the biophysical changes required to remodel them into vesicles, and ESCRT-III-dependent endolysosomal repair seems independent of factors such as PtdIns(3)P or HRS that normally

initiate ILV biogenesis. As well as budded spiral structures that are thought to assemble within membranous necks, ESCRT-III has been observed to form planar spirals on membranes [72,80]. Perhaps a physiological role for these planar spirals is to provide a platform over regions of damaged membrane from which to flow new membrane in for repair (Figure 3iii)?

ESCRTs as regulators of unconventional secretion

Whilst the intraluminal vesicles of MVEs represent an important intermediate in the degradation of membrane bound cargo, MVEs can also fuse with the plasma membrane to release these vesicles as exosomes to the extracellular environment (Figure 1A). Exosomes are but one class of extracellular vesicle but are clinically and physiologically important as they have the potential as biomarkers to report on organismal physiology and pathophysiology, and though cell-cell communication, to influence the behaviour of tissues distal to the site of production [81]. How the cell decides whether to fuse its MVEs with the plasma membrane or with lysosomes, and whether this decision is subject to physiological regulation, is a major outstanding question for the field. However, the small GTPases Rab27a and Rab27b are key mediators of MVE size and docking with the plasma membrane [82] and the target SNAREs Syntaxin-4 and SNAP23 are also required [83]. Although ESCRT-independent routes of sorting cargo to ILVs have been reported [84], ESCRT represents the major determinant of both the biogenesis and cargo loading onto ILVs, and thus onto exosomes [85]. In the absence of proper ESCRT function, both the quality and the quantity of released exosomes will differ. For example, CHMP1A is needed for release of extracellular vesicles containing, amongst other cargoes, the signalling morphogen sonic hedgehog (SHH) [86], paralleling reports that ESCRTs are key mediators of hedgehog release in *D. melanogaster* [87]. Loss of CHMP1A function leads to disrupted SHH signalling and defects in cortical neural progenitor proliferation which may underlie the microcephaly with pontocerebellar hypoplasia observed in patients bearing loss-of-function mutations in CHMP1A [88]. Other cargos can be sorted into ILVs, and thus exosomes, by virtue of interaction with core ESCRT components. The soluble lectin GAL3 contains a P(S/T)AP motif and is incorporated into ILV and exosomes though direct interaction with TSG101 [89], suggesting that this pathway can select soluble cargo. In addition to core ESCRT components, ESCRT-interacting adaptor proteins such as ALIX can also regulate cargo incorporation into

exosomes. A PDZ-domain containing adaptor protein called Syntenin binds ALIX's V-domain by mimicking a LYPxL-type viral late domain [90] and through coordination of PDZ-ligand proteins such as syndecan, these proteins control cargo import into exosomes. ILVs can form in the absence of ALIX, but it seems their quality is altered. For example, PD-L1, a key immunosuppressive molecule that allows many cancers to avoid the immune system, has recently been described to be secreted upon exosomes [91-93] and is incorporated into exosomes in an ALIX-dependent manner [91]. Exosomally secreted PD-L1 could inhibit CD8+ T-cell activation and facilitate melanoma progression, could used as a biomarker to stratify melanoma patients [92] and could be transferred to multiple cell types in the tumour microenvironment to mediate active immunosuppression [93]. Consistent with finding that reduction of ESCRT activity led to enhanced presentation of hedgehog at the plasma membrane in flies [87], depletion of ALIX led to impaired ILV and exosome incorporation of PD-L1 and enhanced surface presentation and immunosuppression in mammalian cancer cells [91]. These data suggest that for MVEs that will fuse with the plasma membrane, ESCRT-dependent sorting from limiting membrane to ILV will determine the balance between exosome and plasma membrane presentation of these secreted cargos (Figure 1A).

ESCRTs have a long history of being peripherally required for autophagy, but only recently has ESCRT-III been demonstrated to play a role in autophagosome closure [94] – a long hypothesised function given the requirement to close a double membraned autophagosome [1]. ESCRTs thus seem necessary for proper autophagic degradation of cargo. Autophagosomes normally fuse with lysosomes for cargo degradation but, like MVEs for exosome secretion, autophagosomes can also fuse with the plasma membrane to effect an unconventional secretory pathway called secretory autophagy [95] Additionally, in *S. cerevisiae* the compartment for unconventional protein secretion (CUPS) pathway generates multilamellar structures that are secreted bypassing the ER in an ESCRT-dependent, but Vps4-independent manner [96]. Whether this pathway exists in higher organisms is unknown, but these data highlight the utility of ESCRT proteins for generating closed membranous structures for atypical secretion pathways.

Perspectives

In recent years, the ESCRT machinery has come to be appreciated as a transplantable membrane remodelling machinery, deployed by the cell in a variety of contexts to effect a toplogically unique membrane fission. Whilst cell biological approaches have told us what this machinery can do, recent advances in biophysical approaches have begun to shed light on the question of exactly how ESCRT proteins catalyse this membrane separation step. Computational models have been helpful in understanding how membrane remodelling can occur, but will likely have to be refined to encompass all the biology we now know ESCRTs are capable of performing. We commonly ascribe a membrane deformation ability to ESCRT-III, based upon the need to remodel the endosomal limiting membrane into an inwardly budding intermediate from which an ILV will be generated. However, it seems that ILV generation may be a topological anomaly and that the majority of situations in which ESCRT-III acts (nuclear envelope sealing, cytokinesis, viral release, autophagosome closure) do not require this deformation as the membranous stalk that ESCRTs are to sever is pre-existing. In the case of viral budding, the L-domain phenotype is budded stalks that can't be severed and it is intuitive to see how a myristolated sphere of Gag will remodel the membrane by itself. Recent anisotropic measurement of sfGFP-Gag shows membrane bending occurs throughout the transition of a flat Gag lattice to a sphere, with ESCRT-III arrival (and departure) only seen immediately prior to scission [18]. Understanding how ESCRT-III polymers transition between flat and 3-dimensional filaments will be important for understanding the biophysical basis for ESCRT-III function and systems to interrogate ESCRT activity within pre-existing stalks [4] will hopefully provide the basis to understanding the elusive molecular basis of this fascinating membrane fission activity.

Figure Legends

Figure 1: Major membrane remodelling pathways performed by the ESCRT machinery in cells. A. Topological equivalence of membrane fission events induced by ESCRT-III in cells including intraluminal vesicle formation upon endosomes (allowing their subsequent release as exosomes when endosomes fuse with the plasma membrane), release of viruses such as HIV-1, sealing of holes in the nuclear envelope (in interphase and in mitosis), repair of plasma (and organellar – see Figure 3) membranes. In addition to these events, ESCRT-III also plays roles in autophagosome closure, neuronal pruning and surveillance of nuclear pore complexes [1]. B. ESCRT-III is thought to assemble as a membrane remodelling 3-dimensional

spiral that severs membrane necks, leading to the separation of previously joined membranes. C. Schematic representation of secondary structural elements in human ESCRT-III proteins. The blocks show helices whose boundaries were determined from a combination of known structures and predicted results. Helices α1-α4 corresponding to the core structural components of ESCRT-III filaments are shown in purple to green colours, downstream regulatory elements are shown in red and yellow as in Bajorek *et al*. (2009) [97]. Additional features, such as C-terminal MIM2 motifs for CHMP4B and CHMP6, or additional helices for IST1, are shown in white blocks. Predictions were obtained by building a multiple sequence alignment of all ESCRT-III primary sequences with MUSCLE [98] and submitting this MSA to the PSIPRED-based Ali2D software [99]. α2 and α3 were predicted as a continuous helices and manually separated based on available crystal structures.

Figure 2: Kinetics and dynamics of ESCRT functions on endosomes. Intraluminal vesicle formation is thought to occur from a stable, clathrin-enriched, microdomain on endosomes. Waves of ESCRT-recruitment underlie the biogenesis of individual ILVs [15]. In plants and worms [16,17], ILVs have been observed to be concatenated and/or tethered by membranes, suggesting that the terminal fission event for ILV release may not be absolutely required during each round of biogenesis.

Figure 3: ESCRT-III-dependent membrane repair. ESCRT-III is proposed to repair minor damage to the plasma membrane and endolysosomes (depicted here). Organellar damage leads to breakdown in compartmentalisation and function. ESCRT-III is recruited to damaged endolysosomes to effect repair. The mechanism of repair is unknown, but may involve: ESCRT-III assembley inside a fenestration in a single membrane (i), damaged regions being incorporated into reverse topology buds for classical ESCRT-III-mediated scission (ii), or planar ESCRT-III assemblies may provide a platform for repair. If repair fails, endolysosomes are targeted for degradation by autophagy.

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Interest Statements

Of exceptional interest from last two years [**]

4, 13, 15, 19, 73, 74

4: Schoneberg et al, Science 2018. In this paper ESCRT-III is shown to exert force on membranes (in a VPS4 and ATP-dependent manner) to effect scission in a topologically correct environment.

13: Mierzwa et al., Nat Cell Biol, 2017. In this paper, Vps4 was demonstrated to act throughout the assembly process to remodel ESCRT-III filaments.

15: Wenzel et al., Nat Commun, 2018. In this paper, ESCRT assembly on endosomes was observed to occur in waves.

19: Adell et al., eLife, 2017. Here, the dynamics of endogenous ESCRT-III recruitment to yeast endosomes was revealed by lattice light sheet imaging

73 and 74: Skowyra et al., Science 2018 and Radulovic et al., EMBO 2018. In these papers, ESCRT-III is shown to have a new role in organelle repair

1

Of significant interest from last 2 years [*]

16, 17, 18, 65, 69, 70,

16 and 17. Frankel et al., Nat Commun (2017) and Buono et al., J Cell Biol (2017). In these papers, it was suggested that intra-endosomal vesicle biogenesis occurred from stable microdomains through repeated rounds of ESCRT activity.

18. Johnson et al., eLife (2018). In this paper, ESCRT-III was shown to be dispensible for membrane bending during HIV-1 egress and to disappear before the final scission.

65. Arii et al., Nat Commun (2018). Here, ESCRT-III was shown necessary for viral traversal of the nuclear envelope, and to play a role in nuclear envelope homeostasis in non-infected cells.

69 and 70. Ruhl et al., (2017) and Gong et al., (2018). In these papers, ESCRT-III was shown to have a protective role to suppress forms of cell death induced through plasma membrane disruption.

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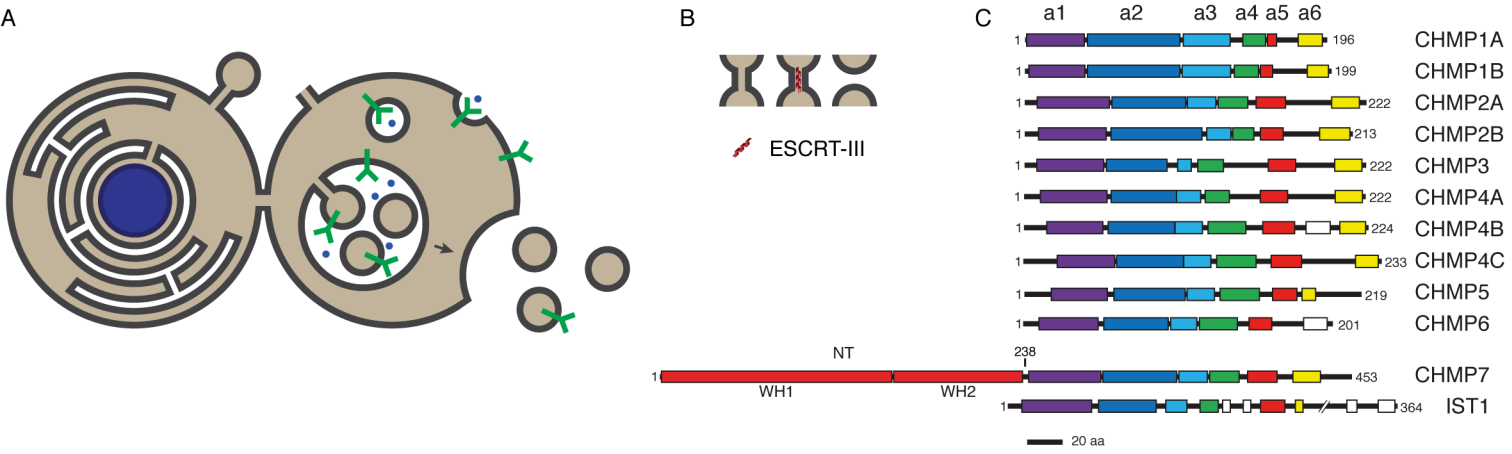
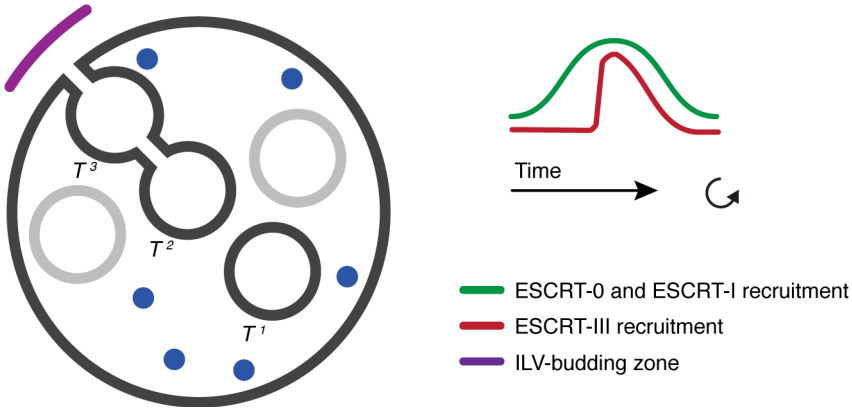
Gatta and Carlton, Figure 1



Gatta and Carlton, Figure 2



Gatta and Carlton, Figure 3

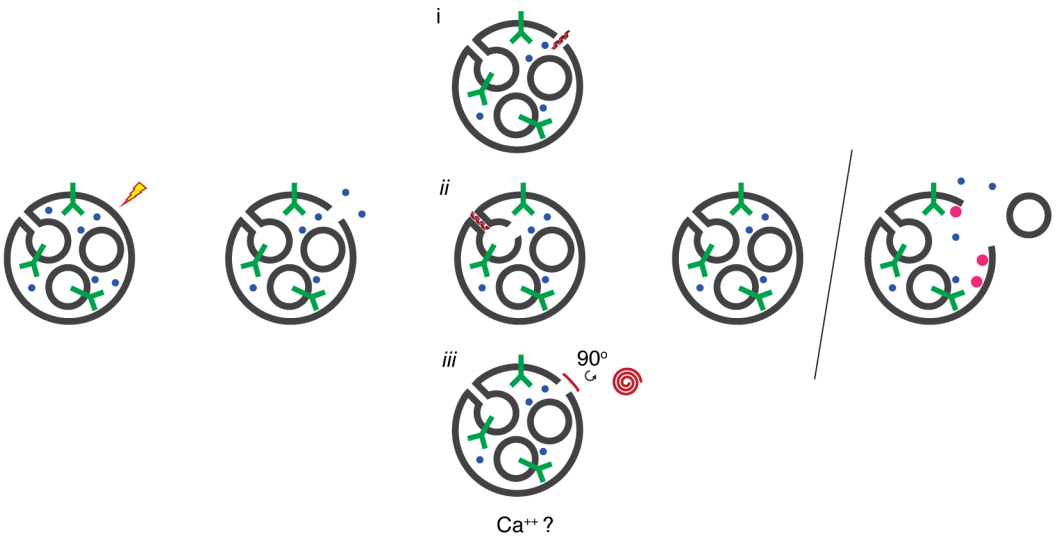


Table 1

	H. sapiens	*Aliases*	*S. cerevisiae*
ESCRT-0	HGS	HRS	Vps27
	STAM1		Hse1
	STAM2		
ESCRT-I	TSG101		Vps23, Stp22
	VPS28		Vps28
	VPS37A		Vps37, Srn2
	VPS37B		
	VPS37C		
	VPS37D		
	UBAP1		Mvb12
	MVB12A		
	MVB12B		
ESCRT-II	VPS25	EAP20	Vps22, Snf8
	VPS22	EAP30	Vps25
	VPS36	EAP45	Vps36
ESCRT-III	CHMP1A		Did2, Vps46,
	CHMP1B		Chm1
	CHMP2A		Did4, Vps2, Chm2
	CHMP2B		
	CHMP3		Vps24, Did3
	CHMP4A		Snf7, Vps32, Did1
	CHMP4B		
	CHMP4C		
	CHMP5		Vps60, Chm5
	CHMP6		Vps20, Chm6
	CHMP7		Chm7
	IST1		Ist1
ESCRT-associated	VPS4A		Vps4
	VPS4B	SKD1	
	VTA1	LIP5, DRG-1	Vta1
	PDCD6IP	ALIX	Bro1, Vps31
	PTPN23	HD-PTP	
	UBPY		Doa4
	STAMBP		